JOURNAL OF BACTERIOLOGY, Mar. 2005, p. 1799–1814 Vol. 187, No. 5
0021-9193/05/$08.00+0 doi:10.1128/JB.187.5.1799–1814.2005

Screening for Quorum-Sensing Inhibitors (QSI) by Use of a Novel Genetic System, the QSI Selector

Thomas Bovbjerg Rasmussen,[1] Thomas Bjarnsholt,[1] Mette Elena Skindersoe,[1] Morten Hentzer,[2] Peter Kristoffersen,[1] Manuela Köte,[3] John Nielsen,[4] Leo Eberl,[3,5] and Michael Givskov[1]*

Center for Biomedical Microbiology, BioCentrum-DTU, Technical University of Denmark, Kgs. Lyngby,[1] Carlsberg Research Center, Biosector, Valby,[2] and Department of Chemistry, Royal Veterinary and Agricultural University of Denmark, Frederiksberg,[4] Denmark; Lehrstuhl für Mikrobiologie, Technische Universitat München, Freising, Germany[3]; and Department of Microbiology, University of Zürich, Zürich, Switzerland[5]

Received 10 April 2004/Accepted 22 October 2004

With the widespread appearance of antibiotic-resistant bacteria, there is an increasing demand for novel strategies to control infectious diseases. Furthermore, it has become apparent that the bacterial life style also contributes significantly to this problem. Bacteria living in the biofilm mode of growth tolerate conventional antimicrobial treatments. The discovery that many bacteria use quorum-sensing (QS) systems to coordinate virulence and biofilm development has pointed out a new, promising target for antimicrobial drugs. We constructed a collection of screening systems, QS inhibitor (QSI) selectors, which enabled us to identify a number of novel QSIs among natural and synthetic compound libraries. The two most active were garlic extract and 4-nitro-pyridine-*N*-oxide (4-NPO). GeneChip-based transcriptome analysis revealed that garlic extract and 4-NPO had specificity for QS-controlled virulence genes in *Pseudomonas aeruginosa*. These two QSIs also significantly reduced *P. aeruginosa* biofilm tolerance to tobramycin treatment as well as virulence in a *Caenorhabditis elegans* pathogenesis model.

Several bacteria show organized behavior when they establish themselves in the eukaryotic host (22). The invading bacteria express a battery of tissue-damaging virulence factors in accordance with their numbers in a process termed quorum sensing (QS) (16). This is accomplished by sensing the concentration of small, diffusible signal molecules produced by the bacteria themselves. In gram-negative bacteria, the signals are *N*-acyl homoserine lactones (AHLs), which are produced by the LuxI family of AHL synthases. The signal molecules differ with respect to the length of their side chains (C4 to C16) and with various degrees of substitution and saturation (34). Short-chain AHLs are freely diffusible over the cell membranes, whereas long-chain AHLs are the substrate of efflux pumps, such as *mexAB-oprM* (36). The AHLs are sensed by proteins belonging to the LuxR family of response regulators. LuxR homologues contain two domains, an AHL binding domain and a DNA binding domain. When AHL is bound, it alters the configuration of the LuxR homologue protein, enabling it to interact with DNA and act as a transcriptional activator (16). It should be noted that some LuxR homologues acts as repressors, blocking transcription in the absence of AHL and, when sufficient AHL is present, derepressing the target gene(s) (6). The two key components of the QS system, the *luxI* and *luxR* homologues, are often linked genes, whereas the QS target genes are localized elsewhere on the genome. In case of *Vibrio fischeri*, the AHL synthase gene itself is a target gene of the QS mechanism, creating an autoinduction loop, which at the triggering (or threshold) AHL concentration gives rise to a burst in AHL production and QS-controlled gene expression.

It has recently become evident that QS target genes are not generally activated at a certain threshold concentration but merely become activated as a continuum at different AHL-cell concentrations (23, 40). *Pseudomonas aeruginosa* utilizes at least two *luxI-luxR* homologous QS systems, *las* and *rhl*, to control expression of virulence factors, including elastase, (alkaline) proteases, rhamnolipids, pyocyanin, and cyanide (37). *P. aeruginosa* is involved in a number of acute and chronic infections of which the fatal pulmonary infection of cystic fibrosis (CF) patients is the best known (46). The significance of QS in relation to infection has become evident in several studies. Erickson et al. (15) have shown that QS signal molecules produced by *P. aeruginosa* can be detected in biologically significant concentrations in sputum from CF patients. Furthermore, another signal molecule, the quinolone signal, has been found in sputum, bronchoalveolar fluid, and mucopurulent fluid from CF patients (8). Using a burned mouse model of infection, Rumbaugh et al. (39) demonstrated that QS-deficient mutants were less lethal then their wild-type counterparts. Apart from taking part in control of expression of virulence factors, AHL signal molecules also interact directly with the host organism. Smith et al. (42) found that 3-oxo-C_{12}-HSL induces COX-2, a membrane-associated prostaglandin E synthase, as well as prostaglandin E_2, resulting in inflammation of the CF lung. Other studies revealed that the 3-oxo-C_{12}-HSL molecules can interfere with components of the immune system such as interleukin-8 and interleukin-12, modulating the response to the presence of *P. aeruginosa* (11, 44).

Due to an increasing number of untreatable, persistent infections, there is a rising need to develop novel strategies which deal with this phenomenon. Such infections often involve the

* Corresponding author. Mailing address: Center for Biomedical Microbiology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark. Phone: 45 4525 2769. Fax: 45 4588 7328. E-mail: immg@pop.dtu.dk.

biofilm mode of growth, which adds to the bacterium's tolerance to conventional antimicrobial treatment (3). Since QS seems to be a key player in regulation of virulence and the formation of tolerant biofilms (9, 25), it is an intriguing target for future antimicrobial chemotherapy.

Several authors have suggested enzymatic degradation of AHLs as a strategy employed by several organisms, including several *Bacillus* species, in minimizing the detrimental effects caused by QS-regulated products (12, 14, 28). *N*-Acyl homoserine lactonase, encoded by *aiiA*, attacks the lactone bond, causing ring opening of AHLs (13). Transgenic plants harboring the *aiiA* gene from *Bacillus thuringiensis* were much less prone to maceration by *Erwinia carotovora*. Two genes, *attM* and *aiiB*, both encoding AHL hydrolases, are found on the Ti plasmid in *Agrobacterium tumefaciens*. As with AiiA, these two enzymes, expressed in *E. carotovora*, reduce the virulence of this bacterium (5). Other bacteria, including *Arthrobacter* sp., *Klebsiella pneumoniae*, *Pseudomonas* sp., *Variovorax* sp., *Comamonas* sp., and *Rhodococcus erythropolis* harbor enzymes capable of AHL destruction (27, 33, 45).

Probably the best-studied example of QS inhibition stems from a marine alga, *Delisea pulchra*. This organism produces several halogenated furanone compounds capable of interfering with AHL-mediated signaling in bacteria (18, 38). The furanones prevent the AHLs from binding to the *luxR* homologues and eventually cause a rapid turnover of these proteins (29, 31). Manefield et al. (30) demonstrated the potential of the halogenated furanones in preventing the pathogen *Vibrio harveyi* from infecting the commercially important black tiger prawn *Paneus monodon*. Furthermore, synthetic furanone derivatives are able to inhibit QS in vitro (23) and attenuate infection by *P. aeruginosa* in vivo (25). The proof of concept delivered by Hentzer et al. (25) has encouraged us to search for nontoxic compounds that might serve as scaffolds for the development of future QS inhibitor (QSI) drugs. To achieve this, we have developed a novel rapid screen for QSIs, which at the same time identifies growth inhibitory substances. The systems are termed QSI selectors (QSIS). When growth is not affected, there is no selection pressure for the development of resistant bacteria. Furthermore, QSI drugs are not expected to eliminate communities of beneficial bacteria present in the host.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO1 was obtained from the *Pseudomonas* genetic stock center (http://www.ecu.edu/pseudomonas; strain PAO0001). The *lasI rhlI* AHL$^-$ PAO-JP strain is deficient in the production of signal molecules (35).

Construction of plasmid pTBR2iB for QSIS1. Plasmid pTBR2iB was constructed as follows. PCR amplification with the primer set P_{luxR_end} (5′-TTAA TTTTTAAAGTATGGGCAATCAATTG-3′) and P_{lux_start} (5′-GCATACTCAT GCTCATAGTTAATTTCTCCTCTTTAA-3′) and with pJBA89 (1) as a template produced a 1,070-bp fragment encoding the *V. fischeri luxR* gene, including the regulatory part of the *lux* operon. The 3′ end of the fragment contains the first 10 bp of the *phlA* gene from *Serratia liquefaciens* (19). A second PCR with the primer set $P_{phlA(F)}$ (5-AACTATGAGCATGAGTATGCCTTTA AGTTTTACCTCTGC-3′) and $P_{phla(R)}$ (5′-GATGCCGAAACCGAGCAGCG-3′) and with pMG330 (19) as a template was performed. This reaction generated a 357-bp fragment encoding a promoterless *phlA* gene. The 5′ end contains the 10 bp immediately upstream of the *luxI* start codon in pJBA89. A third PCR was performed with P_{luxR_end} and $P_{phlA(R)}$ as primers and the fragments from the first two PCRs as a template. The reaction generated a 1,427-bp fragment encoding *luxR* and *phlA*; the latter gene was fused to the promoter region of *luxI* from pJBA89. The fragment was filled in with the Klenow fragment of DNA polymerase I and subsequently cloned into the SmaI site of pUC18not (10) by standard methods, generating pTBR2iB. QSIS1 was created by electrophoration of pTBR2iB into *Escherichia coli* 1100 (*endA thi*) (21).

Construction of plasmid pLasB-SacB1 for QSIS2. A 1.4-kb fragment containing the structural gene *sacB* encoding *Bacillus subtilis* levansucrase (43) flanked by SphI and HindIII sites was PCR amplified with the primers sacB fwd (5′-G CACATGCATGCACATCAAAAAGTTTGC-3′) and sacB rev (5′-GCAAGCT TGCGTTTTTATTTGTTAACTG-3′) and pEX100T as a template (41). The *sacB* fragment was ligated into the corresponding sites of pMHLB2 (23). This produced a fusion, harbored on the *Pseudomonas-E. coli* shuttle vector pUCP22Not (26), between the *P. aeruginosa lasB* promoter and the *B. subtilis sacB* gene, followed by two strong transcriptional terminators from phage λ and the *E. coli rrnB1* operon. Along with the plasB-sacB1 plasmid, this selector strain harbors the pSM1990 plasmid carrying constitutive expressed *lux* genes (non-QS dependent), giving rise to bioluminescence (pSU2007 carrying P_{MT}*luxCDABE*) (32).

Construction of plasmid pPK22 for QSIS3. A promoterless *npt* gene was PCR amplified from pRL1063a (48) with the sense primer 5′-TTGTAAGCTTAAG AGACAGGATGAGGATCG-3′ and antisense primer 5′-TTGTAAGCTTTCA TTTCGAACCCCAGAGTC-3′, both introducing HindIII restrictions sites (underlined). The HindIII-digested PCR product was ligated into the unique HindIII site of the high-copy-number plasmid pJBA25, creating a promoterless *GFP-npt* operon in the plasmid pPK10. pJBA25 is a derivative of pJBA28 (2), with the only difference that pJBA25 contains the *gfp*mut3 instead of *gfp*mut3* in pJBA28. An EcoRI/KpnI fragment from pJBA89 (1), containing the *luxR* gene with its own promoter and upstream of *luxR* with the *luxI* promoter orientated in the opposite direction, was ligated into EcoRI/KpnI digested pPK10, creating pPK11. The coding region of the *E. coli* lambda phage *c*I857 repressor was PCR amplified from pMG300 (21) with the sense primers 5′-CAGGTACCGATTTA ACGTATGAGCAC-3′ and antisense primer 5′-AGGGATCCATTTACTATG TTATGTTCTG-3′ introducing KpnI and BamHI restriction sites, respectively (underlined). The KpnI/BamHI-digested PCR product was ligated into KpnI/BamHI-digested pPK11, creating plasmid pPK12. Due to read-through from the *luxI* promoter into the *GFP-npt* operon in pPK12, the orientation of *luxR-*P_{luxI}*-c*I857 was reversed. First, a derivative of pPK10 was constructed by inserting an EcoRI-BamHI fragment from the pUC18 polylinker into pPK10, creating plasmid pPK20. The *luxR-*P_{luxI}*-c*I857 fragment was isolated from pPK12 as an EcoRI/BamHI fragment, and the 5′ end overhangs were filled in with the Klenow fragment from DNA polymerase I. This blunt-end fragment was ligated into SmaI-digested pPK20, creating the plasmid pPK21. The orientation of the *luxR-*P_{luxI}*-c*I857 fragment in pPK21 was confirmed by restriction digestion analysis.

The *Pr* promoter from the *E. coli* lambda phage *c*I was PCR amplified from pMG300 with the primers 5′-AGGGTACCTCTATCACCGCAAGGG-3′ and 5′-CGGGTACCAGATCTTTAGCTGTCTTGG-3′, both introducing a BamHI site (underlined). The BamHI-digested PCR product was finally ligated into BamHI-digested pPK21, creating the QSIS plasmid pPK22. The correct orientation of the *Pr* promoter in pPK22 was confirmed by monitoring green fluorescent protein (GFP) expression.

Growth medium and conditions. The medium used in this study was either Luria-Bertani (LB) medium (4) or ABT minimal medium supplemented with 0.5% glucose and 0.5% Casamino Acids (AB medium, containing 2.5 mg of thiamine/liter) (7). The medium was supplemented with antibiotics where appropriate. Unless otherwise stated, all strains were incubated at 37°C.

QSIS assays. Preparation of QSIS1 plates was performed in the following way. A total of 250 ml of ABT medium containing 2% agar was melted and cooled to 45°C. 3-Oxo-C_6-HSL (Sigma-Aldrich, Seelze, Germany), ampicillin, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactoside (IPTG) were added in final concentrations of 100 nM, 100 μg/ml, 40 μg/ml, and 100 μM, respectively. After mixing, 1 ml of overnight culture of QSIS1 in ABT supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino Acids was added. Agar plates were subsequently made by pouring 25 ml of the mixture in petri dishes (each, 7 cm in diameter). Preparation of QSIS2 plates was performed the following way. A total of 250 ml of LB agar (2% [wt/vol]) was melted and cooled to 50°C, after which 25 ml of A10 (7) and 14 g of sucrose were added. Gentamicin, 3-oxo-C_{12}-HSL, and C_4-HSL (Sigma) were added to final concentrations of 80 μg/ml, 200 nM, and 200 nM, respectively. After being cooled to 43°C, 5 ml of QSIS2 overnight culture in ABT supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino Acids was added. Plates were poured as described above. Preparation of QSIS3 plates was performed the following way. The selector strain QSIS3 was grown overnight in LB with kanamycin (50 μg/ml) at 30°C. LB with agar was melted and cooled to 45°C. Next, 3-oxo-C_6-HSL was added to a final concentration of 100 nM, kanamycin was

added to a final concentration of 65 μg/ml, and an overnight culture of the selector strain was carried out at a 1,000-fold dilution.

The medium was allowed to solidify, after which wells (4 mm in diameter) were made. A total of 50 μl of test substance was added to each well, and the plates were left for 1 h at room temperature, after which they were incubated overnight at 30°C (QSIS1 and QSIS3) or 37°C (QSIS2). The appearance of a circular growth zone around the reservoir indicated the presence of QSI active compounds in the sample. In case of QSIS1, a blue zone emerged with growth due to the presence of hydrolyzed X-Gal in the plate. During the work with the selector strains, it was noted that a newly outgrown (16 h postinoculation) culture gave the best result with respect to low background growth. As there is a very strong selection for mutations in the genes comprising the selector systems, it is important that incubation times are kept as short as possible.

The pure compounds were tested in concentrations of 10 μM unless otherwise stated. Herbal medicine and food sources were loaded directly if in liquid form or were extracted with 2 volumes (vol/wt) of methanol for 24 h, unless otherwise stated.

Extraction of garlic. Prior to extraction, the stem and dead leaves were removed from the garlic bulbs. A total of 150 g of garlic cloves was shredded with a standard kitchen blender along with 300 ml of toluene. After extraction overnight, the suspension was filtered through Whatman no. 1 filter paper. A total of 150 ml of sterile water was added, and the mixture was stirred for 24 h at room temperature, after which the two phases were allowed to form. The water phase was separated from the organic phase, sterile filtered, and used as raw extract in the present work.

Dose response. To establish a dose-response relationship of a putative QSI compound or a QSI-containing extract, a twofold serial dilution was made with growth medium (ABT with 0.5% [wt/vol] Casamino Acids) in a microtiter dish. Each well contained 100 μl of QSI solution (diluted). Next, 200 μl of an overnight culture (diluted 1:100) of *P. aeruginosa* PAO1 *lasB-gfp*(ASV) (23) was added. Growth was monitored as the optical density at 450 nm (OD_{450}) over a time course of 16 h, and GFP expression was measured at 515 nm.

TLC assay. Two-dimensional thin-layer chromatography (TLC) was performed with RP-18 F_{254S} sheets (Merck). A 50-μl spot of garlic extract was applied to the TLC plate, and the plate was developed. For the first dimension, a methanol:water (60:40) mixture was used. For the second dimension, an ethanol:water (70:30) mixture was used. After elution, the plates were overlaid with 250 ml of ABT agar (2% [wt/vol]) containing 0.5% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids, 100 μg of ampicillin/ml, 100 nM 3-oxo-C_6-HSL, 40 μg of X-Gal/ml, 100 μM IPTG, and 1 ml of QSIS1 overnight culture. The TLC overlay was incubated overnight at 30°C.

DNA array analysis. A total of 200 ml of ABT minimal medium supplemented with 0.5% (wt/vol) Casamino Acids was inoculated with exponentially growing *P. aeruginosa* PAO1 cells ($OD_{600} < 0.5$) to an optical density of 0.05. At a density of 0.7, the culture was split in two 100-ml cultures. The cultures were grown in 500-ml conical flasks in an orbital air shaker operated at 200 rpm at 37°C. Either 2% (vol/vol) garlic extract or 100 μM 4-nitro-pyridine-*N*-oxide (4-NPO) was added to one culture, whereas the second culture served as an untreated control. Treated and untreated cultures showed similar growth. Samples were retrieved at $OD_{600} = 2.0$, mixed with 2 volumes of RNA*later* (Ambion), and stored at −80°C until RNA extraction. RNA extraction was performed with the QIAGEN RNeasy purification kit with the bacterial protocol. To remove all DNA, the purified RNA was treated for 1h with 11 U of DNase I, and RNA was retrieved with the QIAGEN RNeasy purification kit. Synthesis of cDNA was done by mixing 10 μg of RNA with 250 ng of random primers (Invitrogen Life Technologies) in a total volume of 30 μl. The rest of the assay was performed according to the protocol supplied by Affymetrix.

Biofilm. Biofilms were cultivated in continuous-culture once-through flow chambers perfused with sterile ABtrace minimal medium containing 0.3 mM glucose as described previously (23). Garlic extract (1% [vol/vol]) was added to the ABtrace medium where appropriate. Biofilm growth and development were examined by scanning confocal laser microscopy with an LSM 510 system (Carl Zeiss GmbH, Jena, Germany) equipped with an argon laser and a helium-neon laser for excitation of fluorophores. Tolerance of biofilms to tobramycin was assessed by introducing tobramycin (340 μg/ml) to the influent medium to 3-day-old biofilms. After 24 h, biofilms were examined by scanning confocal laser microscopy. Bacterial viability in biofilm cultures was assessed using the LIVE/DEAD BacLight bacterial viability staining kit (Molecular Probes, Inc., Eugene, Oreg.) as described previously (24).

***Caenorhabditis elegans* nematode model.** *P. aeruginosa* strains, PAO1 wt. and PAO1 *lasIrhlI* (23), were grown in 5 ml of LB liquid culture in the presence or absence of 2% (vol/vol) garlic extract overnight with shaking. Similar cultures were made with 100 μM 4-NPO. Culture samples (each, 15 μl) were then spread on BHI agar plates (diameter of each, 55 mm) containing either no or 2% (vol/vol) garlic extract. Likewise, plates containing 100 μM 4-NPO were made. Following overnight incubation at 30°C, five to seven L4 or adult wild-type Bristol N2 worms were transferred to the plates, which then were sealed with parafilm and incubated at 20°C. The number of living worms per plate was determined at various time points with a Stemi SV 6 microscope (Zeiss) at a magnification of ×50. Nematodes were considered dead when they failed to respond to tapping of the plate against the microscope stage. All experiments were carried out five times.

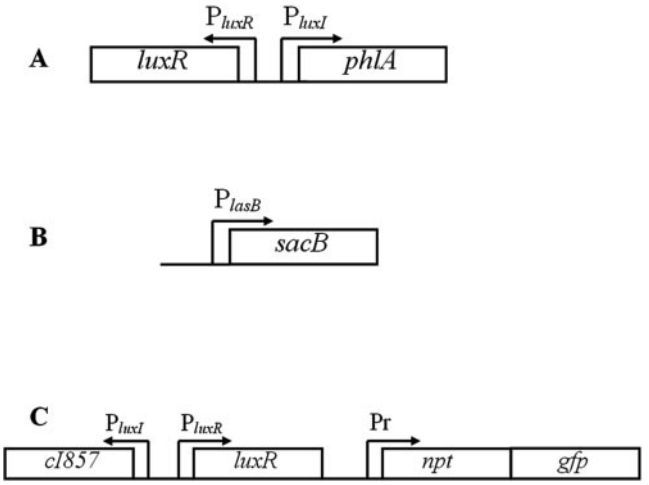

FIG. 1. The three QSIS systems. (A) QSIS1 *phlA* encodes the toxic gene product, expression of which is controlled by LuxR, the system established in *E. coli*. (B) In QSIS2, the LasR- and RhlR-regulated *lasB* promoter control expression of the *sacB* gene, expression of which leads to cell death in the presence of sucrose. The system was established in a *lasI rhlI* mutant of *P. aeruginosa* harboring a plasmid containing a constitutively expressed *lux* operon, which gives rise to expression of bioluminescence in growing cells. (C) The QSIS3 system is also based on LuxR regulation. The *npt* and *gfp* genes, conferring kanamycin resistance and green fluorescence, respectively, are controlled by the *c*I repressor, which in turn is regulated by QS through the *luxI* promoter. The system was established in *E. coli*.

RESULTS AND DISCUSSION

Construction of QSIS. We devised two general types of QSIS systems, A and B (Fig. 1). The basic design of type A comprised a gene encoding a lethal protein (leading to growth arrest and cell death) fused to a QS-controlled promoter. Consequently, type A was unable to grow in the presence of AHL signal molecules unless a functional nontoxic QSI compound was present at a sufficiently high concentration. The basic design of type B employed an antibiotic resistance gene, which was controlled by a repressor. Expression of the repressor was in turn controlled by a QS-regulated promoter. In the presence of AHL, production of the repressor quenched expression of the antibiotic resistance gene, leading to growth inhibition in the presence of the appropriate antibiotic. However, if a QSI compound was present, downregulation of the repressor enabled growth of the bacteria.

QSIS bacteria supplemented with AHL signals were cast in agar plates according to the description in Materials and Methods. Test samples were applied to wells made in the agar. The samples diffused into the agar, creating a gradient with the highest concentration close to the well. The nature of the gradient depended on the molecules in the sample, i.e., large

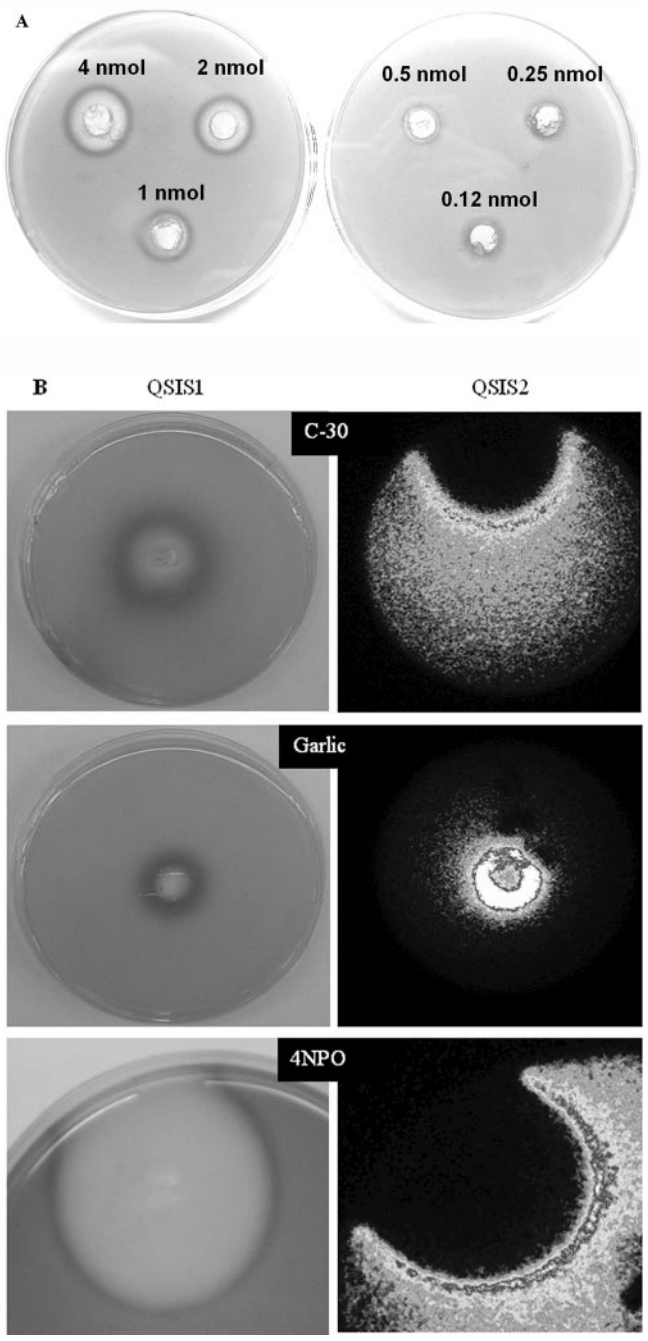

FIG. 2. (A) Calibration of QSIS 1 with different amounts (as indicated) of furanone compound 30. (B) QSIS1 (left) and QSIS2 (right) in action. The QSIS bacteria are cast into the agar along with appropriate AHLs, which activate the killing genes. Test samples as indicated were added to wells in the agar, and the compounds diffused from the well, creating a concentration gradient. Growth of the selector strains was visualized as blue (shown as a dark color, left) for QSIS1 (Lac^+ phenotype on X-Gal agar) or as light emission (shown as a light color, right) for QSIS2 (constitutive bioluminescence expression) with a sensitive charge-coupled device camera.

TABLE 1. Compounds and extracts screened for QSI activity[a]

Sample	QSI
Bean sprout	+
Blackberry	−
Brown onion	−
Chamomile	+
Carrot	+
Coffee	−
Cranberry	−
Poison ivy	−
Garlic	+
Gele Royal	−
Ginseng	−
Habanero	+
Honey	−
Clove	−
Leek	−
Mint tea	−
Propolis	+
Raspberry	−
Red chili	−
Spring onion	−
Tea tree oil	−
Water lily	+
Yellow pepper	+
Blood (plasma)	−
Stinging nettle	−
Anemone	−
Snowberry	−
2-Methyl-imidazole	−
2-Pyrrolidone	−
4-Nitro-pyridine-*N*-oxide	+
3-Hydroxy-benzaldehyde	−
P-Chlorobenzaldehyde	−
2-Phenylcyclohexanone	−
P-Benzoquinone	+
Camphor	−
trans-1,2-Cyclohexandiole	−
Cycloheptane	−
Carbamic acid	−
1,2,3-Benzo-triazole	−
4-Hydroxymethyl-pyridine	−
trans-1,2-Diamino-cyclohexanesulfate	−
2-Phenyl-imidazole	−
2,4,5-Tri-bromo-imidazole	+
2-Mercapto-benzimidazole	−
3,6-Dipyridine	−
1,4-Phenyl-1,2,3-triazole	−
Indole	+
3-Amino-benzen-sulfonamide	−
Benzen-sulfon-amide	−
3-Nitro-benzen-sulfonamide	+
2-Oxazolidione	−
2-Amino-4-methyl-pyridine	−
1,4-Cyclohexan-dione	−
4-Dimethyl-amino-pyridine	−

[a] Various food sources, herbal medicines, and a selection of pure chemical compounds are shown. QSIS1 was employed to screen the samples. +, sample has QSI activity; −, no activity.

molecules diffused more slowly, less-soluble molecules diffused differently from easily solubilized ones, etc. Since the effect of the test compounds on growth of the bacterium is unknown, establishment of this concentration gradient is ideal, since all concentrations are tested at once.

The QSIS1 system was based on the *luxI-gfp* reporter made by Andersen et al. (1). The *luxR* gene and the promoter region of *luxI* were fused to *phlA* from *S. liquefaciens* MG1 (Fig. 1) (21). When expressed without its cognate antidote, PhlB, PhlA

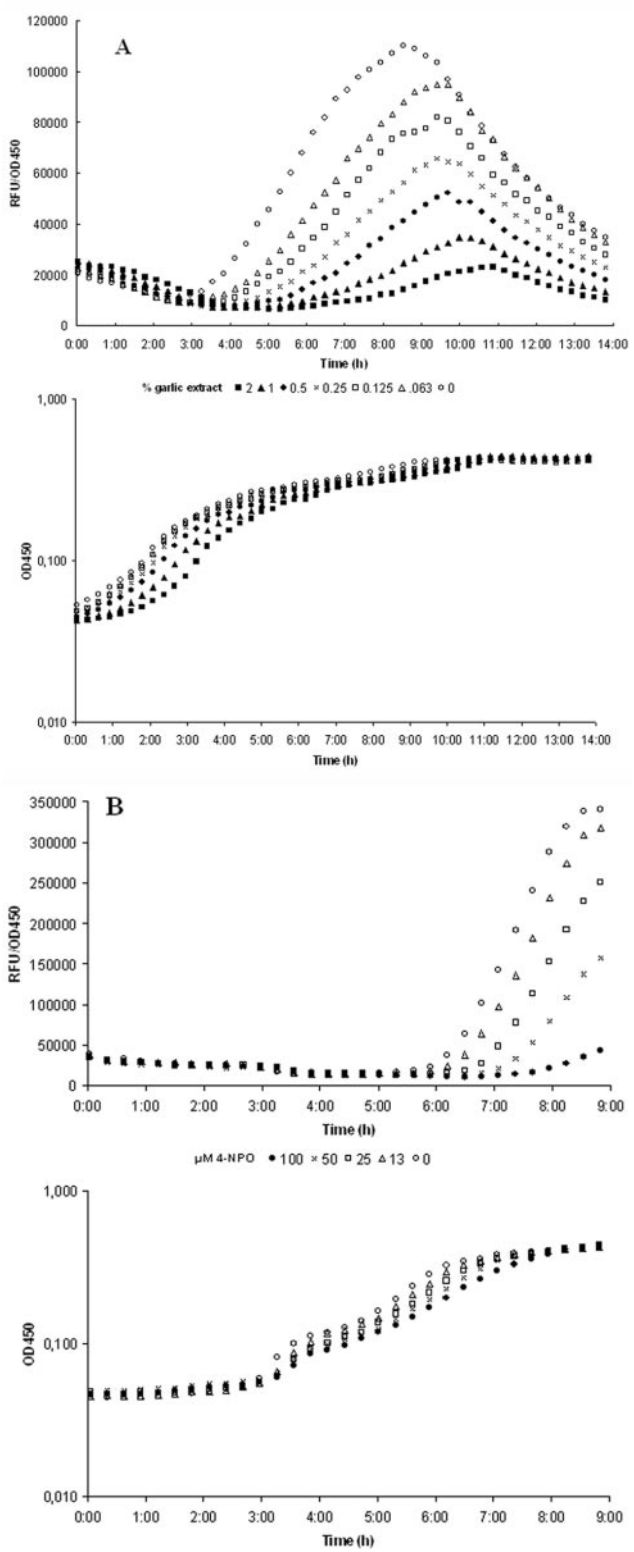

FIG. 3. Dose-response curves of garlic extract (A) and 4-NPO (B). The QS monitor PAO1 (*lasB-gfp*) (23) was incubated with 2% (solid squares), 1% (solid triangles), 0.5% (solid circles), 0.25% (X), 0.13% (open circles), 0.06% (open triangles), or 0% (open circles) (vol/vol) garlic extract. Likewise, the QS monitor was treated with 100 μM (solid circles), 50 μM (X), 25 μM (open squares), 13 μM (open triangles), or 0 μM (open circles) 4-NPO. Growth and fluorescence were followed over time. RFU, relative fluorescence units.

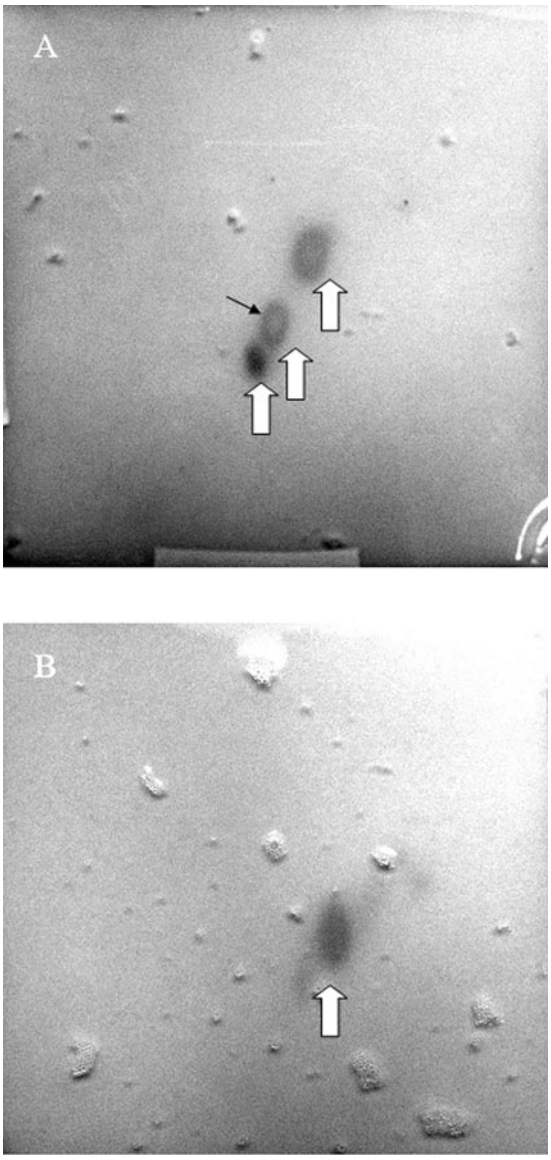

FIG. 4. Two-dimensional TLC of crude toluene extract of garlic (A) and an aqueous phase separated from the toluene (B). Compounds having QSI effect were visualized by overlaying the TLC plate with agar containing QSIS1, X-Gal, and 3-oxo-C_6-HSL. Large arrows indicate areas with growth of strain QSIS1, and the small black arrow points to the center where growth inhibitory activity comigrates with QSI activity.

caused rapid lysis of the host cell (20). In the presence of a QSI compound and 3-oxo-C_6 HSL, the majority of cells survived and grew at the position where the action of the AHL and the QSI counterbalanced each other. The active components of the QSIS1 system were present on an SmaI fragment in pUC18not, which in a *lac*$^+$ *E. coli* background gave rise to a blue circle of growth on X-Gal-supplemented medium.

QSIS2 was based on the *P. aeruginosa* QS systems. The *lasB* promoter was fused to the levansucrase-encoding gene, *sacB*, leading to cell death in the presence of 3-oxo-C_{12}-HSL, C_4-HSL, and sucrose. This fusion is located on a pUCP22not plasmid harbored by a *lasI rhlI* mutant of PAO1 (Fig. 1B). The

TABLE 2. Genes downregulated by two different garlic extracts (G1 and G6) and 4-NPO

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA0012			Hypothetical protein	1.2	−1.0	**−6.8**
PA0049			Hypothetical protein	**−31.7**	**−12.6**	**−76.3**
PA0059	*osmC*	*las* + *rhl*	Osmotically inducible protein OsmC	**−22.2**	**−23.1**	**−51.2**
PA0063			Hypothetical protein	1.3	1.3	**−14.5**
PA0105	*coxB*		Cytochrome *c* oxidase, subunit II	−2.3	−1.0	**−7.9**
PA0106	*coxA*		Cytochrome *c* oxidase, subunit I	−2.1	−1.2	**−5.9**
PA0107			Conserved hypothetical protein	**−14.4**	**−6.2**	**−23.3**
PA0108	*colII*		Cytochrome *c* oxidase, subunit III	**−10.3**	−4.3	**−5.7**
PA0112			Hypothetical protein	**−6.9**	−2.1	**−15.2**
PA0113			Probable cytochrome *c* oxidase assembly factor	−1.7	−1.8	**−19.3**
PA0119			Probable dicarboxylate transporter	−2.8	−1.5	**−7.8**
PA0121			Hypothetical protein	−1.4	−1.3	**−5.6**
PA0122		*las* + *rhl*	Conserved hypothetical protein	−4.5	−4.5	**−14.6**
PA0133			Probable transcriptional regulator	−1.5	1.4	**−14.5**
PA0177			Probable purine binding chemotaxis protein	−2.7	−3.0	**−35.6**
PA0187			Hypothetical protein	−3.8	−4.1	**−16.6**
PA0188			Hypothetical protein	**−28.0**	−3.5	−2.4
PA0215			Probable transporter	−1.0	1.1	**−7.6**
PA0216			Probable transporter	−1,5	−1.3	**−16.4**
PA0224			Probable aldolase	−1.9	−1.2	**−8.5**
PA0229	*pcaT*		Dicarboxylic acid transporter PcaT	**−5.8**	−3.1	−1.7
PA0276			Hypothetical protein	0.0	0.0	**−7.1**
PA0279			Probable transcriptional regulator	−2.1	−1.4	**−12.7**
PA0355	*pfpI*		Protease PfpI	**−14.2**	**−8.4**	**−8.0**
PA0397			Probable cation efflux system protein	−2.5	−1.5	**−10.4**
PA0534			Conserved hypothetical protein	−3.8	−4.0	**−13.2**
PA0567		*las* + *rhl*	Conserved hypothetical protein	**−21.9**	**−14.9**	**−11.4**
PA0699			Probable peptidyl-prolyl *cis-trans* isomerase, PpiC type	0.0	0.0	**−11.4**
PA0755			Probable porin	−2.0	1.3	**−5.6**
PA0797			Probable transcriptional regulator	2.3	1.1	**−9.0**
PA0798	*pmtA*		Phospholipid methyltransferase	−1.7	−1.3	**−15.3**
PA0800			Hypothetical protein	0.0	0.0	**−35.9**
PA0803			Hypothetical protein	−2.3	−2.1	**−17.2**
PA0812			Hypothetical protein	**−5.1**	−1.1	1.2
PA0828			Probable transcriptional regulator	−1.8	−1.1	**−8.0**
PA0846			Probable sulfate uptake protein	1.8	−1.2	**−17.3**
PA0852	*cpbD*	*las* + *rhl*	Chitin binding protein CbpD precursor	**−14.4**	**−6.2**	**−16.9**
PA0873	*phhR*		Transcriptional regulator PhhR	0.0	0.0	**−5.0**
PA0887	*acsA*		Acetyl-coenzyme A synthetase	3.6	1.9	**−5.4**
PA0990			Conserved hypothetical protein	−3.5	**−5.3**	**−9.9**
PA0996	*pqsA*	*las*	Probable coenzyme A ligase	−1.3	1,3	**−7.6**
PA1111			Hypothetical protein	**−5.2**	−5.0	**−6.9**
PA1190			Conserved hypothetical protein	−2.1	−1.6	**−5.2**
PA1202			Probable hydrolase	−1.9	−1.4	**−5.7**
PA1216			Hypothetical protein	−3.4	−2.2	**−13.3**
PA1242			Hypothetical protein	**−6.9**	−3.8	**−38.9**
PA1249	*aprA*	*las* + *rhl*	Alkaline metalloproteinase precursor	−3.4	**−5.1**	**−16.6**
PA1255			Hypothetical protein	−1.1	−1.6	**−9.8**
PA1290			Probable transcriptional regulator	−1.8	−1.5	**−8.6**
PA1297			Probable metal transporter	**−9.8**	**−8.9**	−3.2
PA1298			Conserved hypothetical protein	**−13.3**	−2.7	−2.8
PA1323		*las* + *rhl*	Hypothetical protein	**−26.5**	**−19.5**	**−32.3**
PA1324		*las* + *rhl*	Hypothetical protein	**−22.5**	**−19.1**	**−21.6**
PA1349			Conserved hypothetical protein	−1.9	−2.2	**−5.4**
PA1351			Probable sigma-70 factor, ECF subfamily	−1.8	−2.5	**−21.7**
PA1404			Hypothetical protein	−2.7	−3.2	**−8.8**
PA1412			Hypothetical protein	**−6.1**	−3.7	−3.6
PA1415			Hypothetical protein	−2.3	−2.6	**−12.6**
PA1418			Probable sodium:solute symport protein	−3.4	−2.8	**−5.2**
PA1419			Probable transporter	0.0	0.0	**−9.5**
PA1421	*gbuA*		Guanidinobutyrase	0.0	0.0	**−5.3**
PA1470			Probable short-chain dehydrogenase	−1.9	−1.6	**−5.9**
PA1471			Hypothetical protein	**−6.5**	−4.7	−4.4
PA1475	*ccmA*		Heme exporter protein CcmA	1.4	−1.0	**−13.0**
PA1485			Probable amino acid permease	0.0	0.0	**−11.5**
PA1486			Hypothetical protein	0.0	0.0	**−9.0**

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TABLE 2—*Continued*

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA1514			Conserved hypothetical protein	−3.2	−3.4	**−15.5**
PA1523	*xdhB*		Xanthine dehydrogenase	−2.2	−2.2	**−24.4**
PA1562	*acnA*		Aconitate hydratase 1	−4.3	−3.3	**−6.2**
PA1617			Probable AMP-binding enzyme	−1.6	−2.2	**−7.0**
PA1651			Probable transporter	−1.4	−1.3	**−7.8**
PA1652			Hypothetical protein	−1.2	1.1	**−5.7**
PA1660		*las* + *rhl*	Hypothetical protein	**−8.5**	−2.5	**−10.6**
PA1664		*las* + *rhl*	Hypothetical protein	**−8.6**	−2.1	−4.6
PA1665		*las* + *rhl*	Hypothetical protein	**−5.2**	−3.8	−1.8
PA1667		*las* + *rhl*	Hypothetical protein	**−5.0**	−2.2	−3.4
PA1668			Hypothetical protein	−2.7	−1.7	**−13.9**
PA1669		*las* + *rhl*	Hypothetical protein	**−7.7**	−2.4	−4.6
PA1670	*stp1*		Serine/threonine phosphoprotein phosphatase Stp1	−4.9	−3.3	**−54.3**
PA1700			Conserved hypothetical protein in type III secretion	1.4	1.1	**−14.4**
PA1730			Conserved hypothetical protein	−2.8	−2.6	**−5.6**
PA1763			Hypothetical protein	−1.7	−1.5	**−13.7**
PA1782			Probable serine/threonine-protein kinase	0.0	0.0	**−27.6**
PA1784		*las*	Hypothetical protein	**−5.8**	−4.1	**−5.2**
PA1840			Hypothetical protein	1.4	1.0	**−33.2**
PA1864			Probable transcriptional regulator	−1.7	−2.0	**−13.0**
PA1866			Hypothetical protein	−2.1	−2.1	**−36.0**
PA1869		*las* + *rhl*	Probable acyl carrier protein	−3.1	−1.8	**−8.8**
PA1870			Hypothetical protein	**−7.1**	−2.9	**−5.7**
PA1871	*lasA*	*las* + *rhl*	Las A protease precursor	**−12.9**	**−8.1**	**−65.2**
PA1874			Hypothetical protein	**−5.5**	**−8.0**	**−10.1**
PA1875		*las*	Probable outer membrane protein precursor	**−7.5**	**−7.5**	**−45.8**
PA1876			Probable ATP binding/permease fusion ABC transporter	−4.1	**−5.8**	**−38.3**
PA1881			Probable oxidoreductase	−2.1	−2.8	**−12.6**
PA1887			Hypothetical protein	−2.2	**−6.2**	−3.8
PA1888			Hypothetical protein	−3.2	−3.4	**−5.0**
PA1895			Hypothetical protein	−1.3	−1.3	**−7.9**
PA1901	*phzC2*	*rhl*	Phenazine biosynthesis protein PhzC	**−7.2**	−3.9	**−16.1**
PA1902	*phzD2*	*rhl*	Phenazine biosynthesis protein PhzD	**−7.4**	**−5.1**	**−17.4**
PA1903	*phzE2*	*rhl*	Phenazine biosynthesis protein PhzE	**−10.0**	−4.4	**−11.5**
PA1904	*phzF2*	*rhl*	Probable phenazine biosynthesis protein	**−7.5**	**−5.3**	**−221.1**
PA1905	*phzG2*	*rhl*	Probable pyridoxamine 5′-phosphate oxidase	**−8.2**	−4.7	**−26.2**
PA1914			Conserved hypothetical protein	**−15.8**	**−11.9**	**−33.1**
PA1921			Hypothetical protein	**−34.0**	−3.0	**−18.4**
PA1956			Hypothetical protein	0.0	0.0	**−8.6**
PA1970			Hypothetical protein	−2.3	−1.3	**−6.4**
PA1978			Probable transcriptional regulator	0.0	0.0	**−23.7**
PA1985	*pqqA*		Pyrroloquinoline quinone biosynthesis protein A	−1.8	−2.1	**−7.8**
PA1988	*pqqD*		Pyrroloquinoline quinone biosynthesis protein D	1.1	−1.6	**−11.5**
PA1989	*pqqE*		Pyrroloquinoline quinone biosynthesis protein E	−1.3	−1.9	**−5.3**
PA1999			Probable CoA transferase, subunit A	1.1	−2.3	**−8.6**
PA2000			Probable CoA transferase, subunit B	1.5	−1.7	**−8.0**
PA2001	*atoB*		Acetyl-CoA acetyltransferase	1.5	−1.9	**−9.1**
PA2002			Conserved hypothetical protein	−2.4	**−11.3**	**−39.5**
PA2013			Probable enoyl-CoA hydratase/isomerase	−1.2	1.3	**−8.6**
PA2021			Hypothetical protein	**−13.0**	**−10.6**	**−60.1**
PA2030		*las* + *rhl*	Hypothetical protein	**−6.8**	−4.7	**−18.4**
PA2031		*las* + *rhl*	Hypothetical protein	**−10.8**	−4.4	**−17.3**
PA2035			Probable decarboxylase	0.0	0.0	**−27.6**
PA2046			Hypothetical protein	**−47.2**	**−16.3**	**−31.2**
PA2050			Probable sigma-70 factor, ECF subfamily	2.4	−2.2	**−6.7**
PA2066			Hypothetical protein	−3.9	**−5.2**	**−9.5**
PA2067			Probable hydrolase	**−6.1**	**−5.7**	**−6.9**
PA2068		*rhl*	Probable MFS transporter	**−8.8**	**−5.7**	**−18.6**
PA2069		*rhl*	Probable carbamoyl transferase	**−24.8**	**−11.3**	**−34.1**
PA2084			Probable asparagine synthetase	1.5	−3.2	**−5.3**
PA2086			Probable epoxide hydrolase	1.1	−3.3	**−5.1**
PA2088		*las* + *rhl*	Hypothetical protein	0.0	0.0	**−12.2**
PA2090			Hypothetical protein	−1.2	−4.8	**−8.2**
PA2093			Probable sigma-70 factor, ECF subfamily	−1.6	**−5.4**	−2.0
PA2110			Hypothetical protein	**−13.4**	**−15.7**	**−70.7**

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TABLE 2—*Continued*

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA2111			Hypothetical protein	**−6.9**	**−13.6**	**−14.2**
PA2112			Conserved hypothetical protein	**−18.8**	**−16.1**	**−25.5**
PA2113			Probable porin	**−10.8**	**−6.8**	**−36.0**
PA2114			Probable MFS transporter	**−73.2**	**−15.0**	**−33.5**
PA2116			Conserved hypothetical protein	**−153.1**	**−62.4**	**−87.4**
PA2134			Hypothetical protein	−2.3	−2.4	**−29.8**
PA2137			Hypothetical protein	0.0	0.0	**−33.5**
PA2139			Hypothetical protein	0.0	0.0	**−5.5**
PA2142			Probable short-chain dehydrogenase	**−8.8**	**−6.5**	−4.1
PA2143			Hypothetical protein	**−30.8**	**−8.1**	**−67.1**
PA2144	*glgP*		Glycogen phosphorylase	0.0	0.0	**−40.2**
PA2146			Conserved hypothetical protein	**−7.2**	**−30.9**	**−24.5**
PA2149			Hypothetical protein	−4.8	−3.7	**−8.3**
PA2151			Conserved hypothetical protein	**−92.9**	**−78.8**	**−24.2**
PA2153	*glgB*		1,4 α-Glucan branching enzyme	**−9.5**	**−6.6**	**−26.0**
PA2158			Probable alcohol dehydrogenase (Zn dependent)	−4.5	−2.7	**−17.7**
PA2159			Conserved hypothetical protein	−1.1	−1.8	**−7.9**
PA2162			Probable glycosyl hydrolase	−2.8	−4.3	**−12.4**
PA2163			Hypothetical protein	−3.1	−4.7	**−17.9**
PA2165			Probable glycogen synthase	−1.5	−2.1	**−5.6**
PA2166			Hypothetical protein	**−5.3**	**−6.3**	**−10.6**
PA2167			Hypothetical protein	**−12.2**	**−6.8**	**−126.9**
PA2168			Hypothetical protein	−2.5	−4.3	**−12.4**
PA2169			Hypothetical protein	−4.1	**−5.9**	−3.0
PA2170			Hypothetical protein	**−9.3**	**−7.6**	−2.1
PA2171			Hypothetical protein	−3.0	−2.2	**−9.4**
PA2172			Hypothetical protein	−3.3	**−5.5**	−3.0
PA2174			Hypothetical protein	−3.7	−3.1	**−5.9**
PA2175			Hypothetical protein	−3.2	−3.8	**−25.5**
PA2176			Hypothetical protein	**−13.2**	**−6.5**	**−143.6**
PA2184			Conserved hypothetical protein	−3.5	−3.1	**−65.9**
PA2187			Hypothetical protein	−3.2	−1.3	**−42.8**
PA2190			Conserved hypothetical protein	**−5.1**	−4.6	**−18.0**
PA2191	*exoY*		Adenylate cyclase ExoY	−1.2	−1.1	**−5.6**
PA2198			Hypothetical protein	−1.9	−1.7	**−21.2**
PA2225			Hypothetical protein	−2.2	−1.3	**−10.2**
PA2226			Hypothetical protein	−1.6	−1.8	**−5.5**
PA2244			Hypothetical protein	−3.5	**−5.4**	−3.3
PA2245			Hypothetical protein	**−14.1**	−3.8	**−15.6**
PA2250	*lpdV*		Lipoamide dehydrogenase-Val	−1.0	1.1	**−5.7**
PA2251			Hypothetical protein	−1.2	1.0	**−8.6**
PA2272	*pbpC*		Penicillin binding protein 3A	0.0	0.0	**−19.9**
PA2277	*arsR*		ArsR protein	1.2	−1.0	**−12.1**
PA2278	*arsB*		ArsB protein	−2.0	−1.2	**−13.1**
PA2296			Hypothetical protein	1.1	−1.6	**−10.1**
PA2300	*chiC*	*rhl*	Chitinase	**−37.1**	**−20.5**	**−32.4**
PA2317			Probable oxidoreductase	−1.3	−1.4	**−10.6**
PA2322			Gluconate permease	−2.9	−2.4	**−49.5**
PA2414		*las* + *rhl*	L-sorbosone dehydrogenase	**−14.8**	**−8.7**	**−7.0**
PA2415		*las* + *rhl*	Hypothetical protein	**−13.2**	**−5.7**	**−9.4**
PA2433		*las* + *rhl*	Hypothetical protein	**−41.8**	**−28.9**	**−30.8**
PA2448			Hypothetical protein	−3.5	−3.3	**−35.8**
PA2457			Hypothetical protein	−1.4	−1.3	**−9.8**
PA2485			Hypothetical protein	**−8.9**	**−10.4**	**−6.8**
PA2486			Hypothetical protein	**−6.9**	**−6.4**	**−5.0**
PA2505			Probable porin	−1.5	−1.2	**−8.1**
PA2528			Probable RND efflux membrane fusion protein precursor	−1.1	−1.5	**−5.3**
PA2534			Probable transcriptional regulator	−1.4	−1.7	**−6.3**
PA2557			Probable AMP binding enzyme	**−9.0**	**−6.6**	**−6.9**
PA2564		*las* + *rhl*	Hypothetical protein	−2.5	**−18.8**	−1.9
PA2570	*palL*	*rhl*	PA-I galactophilic lectin	**−50.6**	**−16.9**	**−19.0**
PA2598			Hypothetical protein	2.9	−1.8	**−20.7**
PA2602			Hypothetical protein	1.5	−1.2	**−10.8**
PA2692			Probable transcriptional regulator	−2.2	1.3	**−5.3**
PA2696			Probable transcriptional regulator	−1.5	−1.1	**−10.4**
PA2698			Probable hydrolase	−1.5	−1.1	**−12.6**

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TABLE 2—*Continued*

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA2701			Probable MFS transporter	1.2	−1.9	**−6.2**
PA2708			Hypothetical protein	−2.3	−3.4	**−98.8**
PA2717	*cpo*		Chloroperoxidase precursor	−4.2	−4.9	**−39.8**
PA2722			Hypothetical protein	−2.7	−2.2	**−7.9**
PA2746			Hypothetical protein	−4.2	−2.7	**−18.6**
PA2747		*las* + *rhl*	Hypothetical protein	**−18.8**	**−16.6**	**−11.5**
PA2751			Conserved hypothetical protein	−5.0	−3.4	**−10.8**
PA2754			Conserved hypothetical protein	**−10.7**	**−8.9**	−4.8
PA2777			Conserved hypothetical protein	**−6.7**	**−6.9**	**−14.1**
PA2819			Hypothetical protein	−0.0	0.0	**−5.2**
PA2862	*lipA*		Lactonizing lipase precursor	−1.4	−1.6	**−6.3**
PA2893			Probable very-long-chain acyl-CoA synthetase	−1.6	−1.2	**−5.8**
PA2895			Hypothetical protein	−2.3	−3.8	**−11.1**
PA2909			Hypothetical protein	1.0	−1.3	**−11.1**
PA2937			Hypothetical protein	−3.8	−3.9	**−13.7**
PA2939		*las*	Probable aminopeptidase	−4.7	−3.2	**−38.9**
PA3019			Probable ATP binding component of ABC transporter	1.2	−1.2	**−6.8**
PA3025			Probable FAD dependent glycerol-3-phosphate dehydrogenase	−1.5	−1.1	**−8.0**
PA3042			Hypothetical protein	−1.8	−4.0	**−8.2**
PA3181			2-Keto-3-deoxy-6-phosphogluconate aldolase	−1.4	−1.6	**−8.8**
PA3183	*zwf*		Glucose-6-phosphate 1-dehydrogenase	−2.2	−3.2	**−5.1**
PA3231			Hypothetical protein	**−11.8**	**−12.0**	**−24.7**
PA3234			Probable sodium:solute symporter	**−13.2**	**−13.9**	**−5.3**
PA3235			Conserved hypothetical protein	0.0	0.0	**−13.4**
PA3250			Hypothetical protein	−1.7	−1.7	**−6.2**
PA3251			Hypothetical protein	−2.4	−2.3	**−6.0**
PA3271			Probable two-component sensor	−1.3	−1.6	**−21.5**
PA3273			Hypothetical protein	−4.5	**−5.9**	**−14.4**
PA3274		*las* + *rhl*	Hypothetical protein	**−15.6**	**−7.3**	**−58.6**
PA3316			Probable permease of ABC transporter	−1.7	−1.2	**−12.5**
PA3324			Probable short-chain dehydrogenase	0.0	0.0	**−9.9**
PA3332		*rhl*	Conserved hypothetical protein	−1.7	1.6	**−5.7**
PA3333	*fabH2*	*rhl*	3-Oxoacyl-[acyl-carrier-protein] synthase III	−1.5	1.3	**−10.5**
PA3336		*rhl*	Probable MFS transporter	−1.7	1.5	**−6.7**
PA3361		*rhl*	Hypothetical protein	**−8.7**	**−7.0**	**−46.9**
PA3365			Probable chaperone	0.0	0.0	**−18.0**
PA3366	*amiE*		Aliphatic amidase	−1.7	−2.0	**−6.8**
PA3369			Hypothetical protein	**−5.4**	**−6.4**	**−41.8**
PA3370			Hypothetical protein	**−10.1**	**−12.3**	**−23.7**
PA3371		*las* + *rhl*	Hypothetical protein	**−9.8**	**−9.5**	**−84.9**
PA3416			Probable pyruvate dehydrogenase E1 component, beta chain	−4.1	**−6.3**	**−5.8**
PA3451			Hypothetical protein	−2.7	−3.7	**−9.1**
PA3459			Probable glutamine amidotransferase	**−9.7**	**−5.3**	−4.3
PA3460			Probable acetyltransferase	**−9.9**	**−5.7**	**−14.0**
PA3461			Conserved hypothetical protein	**−6.0**	**−7.3**	**−8.6**
PA3466			Probable ATP-dependent RNA helicase	1.1	1.1	**−7.9**
PA3478	*rhlB*	*las* + *rhl*	Rhamnosyltransferase chain B	**−10.5**	**−7.1**	**−44.0**
PA3479	*rhlA*	*las* + *rhl*	Rhamnosyltransferase chain A	**−9.6**	**−7.2**	**−29.1**
PA3492			Conserved hypothetical protein	−3.0	−1.7	**−5.5**
PA3500			Conserved hypothetical protein	−1.8	−1.8	**−7.6**
PA3520		*las* + *rhl*	Hypothetical protein	**−8.5**	**−9.1**	**−87.3**
PA3562			Probable phosphotransferase system enzyme I	−1.7	−1.8	**−13.2**
PA3581	*glpF*		Glycerol uptake facilitator protein	0.0	0.0	**−76.5**
PA3584	*glpD*		Glycerol-3-phosphate dehydrogenase	−1.9	−1.4	**−9.2**
PA3691		*las* + *rhl*	Hypothetical protein	**−13.4**	**−9.4**	**−12.8**
PA3692		*las* + *rhl*	Probable outer-membrane protein precursor	**−22.3**	**−14.2**	**−14.0**
PA3706			Probable protein methyltransferase	−1.4	−1.7	**−5.3**
PA3710			Probable GMC-type oxidoreductase	0.0	0.0	**−10.2**
PA3724	*lasB*	*las* + *rhl*	Elastase LasB	**−6.8**	−3.1	**−22.6**
PA3734			Hypothetical protein	**−7.3**	−5.0	**−32.9**
PA3739			Probable sodium/hydrogen antiporter	−1.5	−1.4	**−22.3**
PA3758			Probable *N*-acetylglucosamine-6-phosphate deacetylase	−3.1	−2.1	**−6.0**
PA3779			Hypothetical protein	−1.7	−2.0	**−5.1**
PA3788			Hypothetical protein	**−8.4**	**−7.6**	**−6.5**
PA3815			Conserved hypothetical protein	−2.1	−2.1	**−9.8**
PA3819			Conserved hypothetical protein	**−5.2**	**−5.3**	−3.1

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TABLE 2—*Continued*

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA3845			Probable transcriptional regulator	−1.7	−1.6	**−17.1**
PA3887	*nhaP*		Na^+/H^+ antiporter NhaP	1.0	1.1	**−17.2**
PA3888			Probable permease of ABC transporter	**−7.1**	**−8.6**	**−8.7**
PA3889			Probable binding protein component of ABC transporter	−4.8	**−6.7**	**−10.5**
PA3890		*las* + *rhl*	Probable permease of ABC transporter	**−5.6**	**−5.7**	**−95.6**
PA3898			Probable transcriptional regulator	1.2	−1.4	**−18.3**
PA3920			Probable metal transporting P-type ATPase	−1.3	−1.8	**−28.4**
PA3923			Hypothetical protein	−1.6	−1.0	**−9.0**
PA3957			Probable short-chain dehydrogenase	−3.6	−1.8	**−5.9**
PA4078			Probable nonribosomal peptide synthetase	**−7.2**	**−5.2**	**−8.0**
PA4086	*cupB1*		Probable fimbrial subunit CupB1	−1.6	1.1	**−11.2**
PA4106			Conserved hypothetical protein	−1.7	−1.1	**−5.5**
PA4129		*las* + *rhl*	Hypothetical protein	−2.6	**−6.5**	**−7.2**
PA4130		*las* + *rhl*	Probable sulfite or nitrite reductase	−2.1	**−8.3**	**−13.2**
PA4133		*las* + *rhl*	Cytochrome c oxidase subunit (cbb3 type)	**−5.6**	**−11.5**	**−123.5**
PA4134		*las* + *rhl*	Hypothetical protein	−3.4	−3.5	**−5.4**
PA4138	*tyrS*		Tyrosyl-tRNA synthetase	0.0	0.0	**−11.7**
PA4141		*las* + *rhl*	Hypothetical protein	**−6.6**	−4.4	**−23.7**
PA4142		*las* + *rhl*	Probable secretion protein	**−7.2**	**−5.6**	**−33.9**
PA4143			Probable toxin transporter	−4.7	−3.5	**−148.3**
PA4171			Probable protease	−4.7	−2.5	**−16.8**
PA4172			Probable nuclease	**−6.4**	−3.4	**−7.0**
PA4175	*prpL*	*las*	Pvds-regulated endoprotease, lysyl class	**−6.9**	−4.2	**−20.7**
PA4199			Probable acyl-CoA dehydrogenase	−1.7	−1.2	**−9.2**
PA4204			Conserved hypothetical protein	**−7.6**	−3.2	−4.5
PA4205	*mexG*		Hypothetical protein	−4.1	−3.7	**−6.0**
PA4207	*mexI*		Probable RND efflux transporter	−4.1	**−5.3**	−3.3
PA4209	*phzM*	*rhl*	Probable phenazine-specific methyltransferase	−4.2	−3.1	**−17.1**
PA4210	*phzA1*	*rhl*	Probable phenazine biosynthesis protein	**−5.6**	−2.7	**−15.17**
PA4211	*phzB1*	*rhl*	Probable phenazine biosynthesis protein	**−6.4**	−3.5	**−25.0**
PA4217	*phzS*	*rhl*	Flavin-containing monooxygenase	**−5.3**	−2.7	**−15.4**
PA4290			Probable chemotaxis transducer	−4.4	**−8.1**	**−27.1**
PA4294			Hypothetical protein	−2.0	−2.7	**−5.5**
PA4300			Hypothetical protein	−2.8	−3.5	**−5.7**
PA4302			Probable type II secretion system protein	−3.6	**−8.5**	−3.2
PA4303			Hypothetical protein	−2.5	**−6.0**	**−6.0**
PA4304			Probable type II secretion system protein	−2.6	−3.5	**−5.2**
PA4305			Hypothetical protein	−3.4	−4.3	**−21.1**
PA4306		*las* + *rhl*	Hypothetical protein	**−5.7**	**−10.1**	**−20.1**
PA4342			Probable amidase	1.0	−1.0	**−25.8**
PA4345			Hypothetical protein	−4.9	**−5.7**	**−10.8**
PA4346			Hypothetical protein	−1.4	−2.3	**−9.0**
PA4354			Conserved hypothetical protein	−1.7	−1.4	**−28.4**
PA4377			Hypothetical protein	−4.7	**−6.1**	**−15.9**
PA4391			Hypothetical protein	−1.5	−1.6	**−7.1**
PA4397	*panE*		Ketopantoate reductase	−1.4	−1.5	**−6.1**
PA4435			Probable acyl-CoA dehydrogenase	−1.0	1.4	**−5.4**
PA4456			Probable ATP-binding component of ABC transporter	1.5	1.3	**−5.9**
PA4573			Hypothetical protein	−4.1	**−5.6**	−4.4
PA4648			Hypothetical protein	**−5.2**	**−8.8**	**−11.9**
PA4649			Hypothetical protein	−3.7	**−8.7**	**−20.9**
PA4650			Hypothetical protein	**−5.4**	−4.3	**−31.2**
PA4651			Probable pilus assembly chaperone	−3.4	**−5.7**	−4.3
PA4653			Hypothetical protein	−2.0	−1.8	**−8.5**
PA4738		*las* + *rhl*	Conserved hypothetical protein	**−5.4**	**−7.2**	**−76.7**
PA4739		*las* + *rhl*	Conserved hypothetical protein	**−11.4**	**−11.3**	**−28.1**
PA4785			Probable acyl-CoA thiolase	−4.8	−4.5	**−10.1**
PA4786			Probable short-chain dehydrogenase	−3.2	−3.3	**−12.0**
PA4788			Hypothetical protein	−1.7	−1.7	**−13.9**
PA4816			Hypothetical protein	−4.1	−1.8	**−11.4**
PA4828			Conserved hypothetical protein	−1.3	1.1	**−8.6**
PA4829	*lpd3*		Dihydrolipoamide dehydrogenase 3	−1.3	−1.7	**−14.3**
PA4835			Hypothetical protein	**−11.9**	−2.3	−2.1
PA4869			Hypothetical protein	−2.5	−2.2	**−15.9**
PA4876	*osmE*		Osmotically inducible lipoprotein OsmE	**−13.5**	**−12.9**	**−10.2**
PA4877			Hypothetical protein	−4.0	**−5.7**	−3.9

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TABLE 2—*Continued*

PA gene[a]	Gene	Regulation	Description[b]	Fold change[c]		
				G1	G6	4-NPO
PA4880			Probable bacterioferritin	−3.7	−4.2	**−14.2**
PA4908			Hypothetical protein	0.0	0.0	**−8.4**
PA4910			Probable ATP-binding component of ABC transporter	−1.5	−1.2	**−18.7**
PA4980			Probable enoyl-CoA hydratase/isomerase	1.0	−1.0	**−14.4**
PA5034	*hemE*		Uroporphyrinogen decarboxylase	0.0	0.0	**−6.1**
PA5061			Conserved hypothetical protein	−4.1	−3.7	**−7.2**
PA5093			Probable histidine/phenylalanine ammonia-lyase	0.0	0.0	**−5.5**
PA5096			Probable binding protein component of ABC transporter	0.0	0.0	**−12.8**
PA5098	*hutH*		Histidine ammonia-lyase	−2.9	**−6.6**	−2.6
PA5099			Probable transporter	0.0	0.0	**−21.9**
PA5100	*hutU*		Urocanase	1.6	−3.2	**−5.2**
PA5155			Probable permease of ABC transporter	1.0	1.5	**−8.3**
PA5171	*arcA*		Arginine deiminase	**−5.7**	−2.6	1.5
PA5172	*arcB*		Omithine carbamoyltransferase, catabolic	**−6.4**	−2.7	1.4
PA5173	*arcC*		Carbamate kinase	**−7.6**	−4.5	−1.3
PA5212			Hypothetical protein	**−8.4**	**−8.7**	**−5.8**
PA5235	*glpT*		Glycerol-3-phosphate transporter	**−5.7**	−2.1	**−13.3**
PA5297	*paxB*		Pyruvate dehydrogenase (cytochrome)	−4.7	−3.7	**−6.6**
PA5352			Conserved hypothetical protein	−1.3	−1.3	**−5.1**
PA5383			Conserved hypothetical protein	**−5.7**	−3.1	**−5.9**
PA5457			Hypothetical protein	1.0	1.0	**−17.0**
PA5460			Hypothetical protein	**−6.8**	−2.8	−2.0
PA5468			Probable citrate transporter	−1.9	1.2	**−6.7**
PA5481		*las* + *rhl*	Hypothetical protein	**−5.8**	**−12.2**	**−79.3**
PA5482		*las* + *rhl*	Hypothetical protein	**−22.7**	**−16.6**	**−77.0**
PA5503			Probable ATP binding component of ABC transporter	1.8	−1.9	**−5.4**
PA5506			Hypothetical protein	**−5.0**	−3.1	−4.5
PA5517			Conserved hypothetical protein	−1.1	−1.1	**−13.6**
PA5541			Probable dihydroorotase	−2.4	−1.1	**−11.6**
PA5544			Conserved hypothetical protein	**−5.5**	−2.5	−1.3

[a] Genes marked with boldface type were found to be QS regulated (25).
[b] Description from the *Pseudomonas* genome project (http://www.pseudomonas.com). CoA, coenzyme A; MFS, major facilitator superfamily.
[c] Values are fold change in hybridization signal comparing a treated and untreated planktonic culture at OD_{600} = 2.0. Boldface type indicates a change of fivefold or higher. LasR/RhlR regulation is defined by Hentzer et al. (25).

presence of a QSI compound rescued the cells, and a circle of growth arose at the position where the action of the AHL signals and the QSI counterbalanced each other. As an additional feature, we equipped the cells with bioluminescence for detection sensitivity.

In the presence of 3-oxo-C_6-HSL, the QSIS3 system achieved repression of the *npt* gene by the *luxR-*P_{luxI} quorum sensor (Fig. 1C). In the presence of a QSI, derepression of antibiotic resistance led to growth of the selector strain. We used the *c*I857 repressor and the corresponding *Pr* promoter to control the *npt* gene. As an additional feature of the selector, we inserted a promoterless *gfp* gene immediately downstream of the *npt* gene.

Calibration of the selector systems. The halogenated furanones were indeed active with all three selector systems described. We used furanone 30 (25) and furanone 56 (23) to calibrate each system (Fig. 2A). The QSIS1 assay was optimized with respect to the concentration of 3-oxo-C_6-HSL and inoculum size, as described in Material and Methods. The detection limit for furanone compound 30 with QSIS1 was 500 pmol.

QSIS2 was established in the *lasI rhlI* mutant of *P. aeruginosa*, and the assay was optimized with respect to C_4-HSL and 3-oxo-C_{12}-HSL, as well as to sucrose content and inoculum size (see Materials and Methods). We noted some false-positive results with this selector system. For example, high concentrations of glucose resulted in a strong positive result, probably due to interference with the sucrase killing system. Therefore, if QSIS2 is used to screen extracts that contain carbohydrates (such as extracts from fruit or berries) the results should be verified by QSIS1 or QSIS3.

The assay conditions for QSIS3 were optimized for kanamycin and 3-oxo-C_6-HSL (see Materials and Methods). In the absence of AHL, the selector strain was able to grow in the presence of kanamycin in the range of 50 to 100 μg/ml. In the presence of both kanamycin and 3-oxo-C_6-HSL (50 to 100 nM), the selector strain was unable to grow in LB agar at 30°C, but if the furanone compounds were added to the reservoir, growth was restored (data not shown).

Screening of extracts and/or compounds. Since the recombinant *lux* QS system responds to a broad spectrum of signal molecules (2), we speculated that it would also respond to a broad spectrum of QSIs. Therefore, QSIS1 was used as the first screen. The *las* system responded almost exclusively to 3-oxo-C_{12}-HSL (23), making it a more narrow-range screening system. Both QSIS1 and QSIS3 are based on the *lux* system; hence, they essentially screen for similar events—inhibition of LuxR activation. Since QSIS3 relies on the temperature-sensitive *c*I repressor, it is more difficult to handle in routine screenings and was therefore not used further in the present study.

We screened two different compound libraries; the first was

TABLE 3. Genes upregulated by two different garlic extracts (G1 and G6) and 4-NPO

PA gene[a]	Gene	Regulation	Description[b]	Fold Change[c]		
				G1	G6	4-NPO
PA0047			Hypothetical protein	3.0	2.6	**5.8**
PA0083			Conserved hypothetical protein	2.5	3.3	**5.8**
PA0085			Conserved hypothetical protein	3.1	3.6	**8.8**
PA0197			Hypothetical protein	**8.1**	−1.6	−1.9
PA0198	*exbB1*		Transport protein ExbB	**6.2**	−2.1	−2.5
PA1556			Probable cytochrome *c* oxidase subunit	**5.4**	1.8	**11.0**
PA1557			Probable cytochrome oxidase subunit (cbb3 type)	3.3	1.6	**8.3**
PA1673			Hypothetical protein	2.0	1.8	**18.1**
PA1715	*pscB*		Type III export apparatus protein	**7.4**	**5.4**	4.6
PA1718	*pscE*		Type III export protein PscE	4.4	2.7	**6.5**
PA1746			Hypothetical protein	1.1	−1.1	**7.1**
PA2285			Hypothetical protein	−1.1	−1.1	**5.4**
PA2310			Hypothetical protein	**5.9**	−3.2	1.0
PA3283			Conserved hypothetical protein	2.4	2.4	**25.4**
PA3284			Hypothetical protein	2.0	2.0	**12.1**
PA3441		*las* + *rhl*	Probable molybdopterin binding protein	**5.9**	−2.5	−2.5
PA3442			Probable ATP-binding component of ABC transporter	**9.2**	−1.9	−2.5
PA3444			Conserved hypothetical protein	**8.3**	1.1	−1.1
PA3445			Conserved hypothetical protein	**5.3**	−1.1	1.3
PA3570	*mmsA*		Methylmalonate-semialdehyde dehydrogenase	**7.6**	2.4	−3.0
PA3720			Hypothetical protein	**10.2**	3.2	1.1
PA3935	*tauD*		Murine dioxygenase	**8.4**	−1.3	1.4
PA3938			Probable periplasmic taurine binding protein precursor	**6.2**	−1.3	−1.2
PA4067	*oprG*		Outer membrane protein OprG precursor	2.3	1.6	**5.9**
PA4191			Probable iron-ascorbate oxidoreductase	**5.9**	1.2	1.0
PA4195			Probable binding protein component of ABC transporter	**7.1**	1.7	−1.1
PA4710	*phuR*		Hemoglobin uptake outer membrane receptor PhuR precursor	4.6	**5.5**	2.7
PA5027			Hypothetical protein	1.3	1.8	**6.3**
PA5054	*hslU*		Heat shock protein HslU	4.4	**5.4**	2.3
PA5083			Conserved hypothetical protein	**5.8**	2.3	1.2
PA5475			Hypothetical protein	−1.0	−1.2	**5.4**

[a] Gene marked with boldface were found to be QS regulated (25).
[b] Description from the *Pseudomonas* genome project (http://www.pseudomonas.com).
[c] Values are fold changes in hybridization signal comparing a treated and untreated planktonic culture at OD_{600} = 2.0; data in boldface indicate a change of fivefold or higher. LasR-RhlR regulation is defined by Hentzer et al. (25).

based on extracts of several plants and herbs, and the second consisted of various pure chemical compounds (Table 1). Plants and herbs were extracted overnight with methanol, which does not in itself exhibit QSI activity in the screens (data not shown). The most active samples from the two groups, garlic and 4-NPO, were chosen for further studies (Fig. 2B). Extraction of garlic with different solvents revealed that toluene gave the most active extract (data not shown). Toluene did not exhibit QSI activity in our screens (data not shown). The crude toluene extract of garlic used in the initial screen exerted a strong growth inhibitory effect in addition to QSI activity. This is probably due to the high amount of allicin produced when garlic is crushed. We devised an extraction protocol to avoid most of the growth inhibitory compounds (see Materials and Methods). Extraction into an aqueous phase significantly reduced the growth inhibitory effect. We also tested pure, synthetic allicin and found that it did not give rise to a positive result with our systems (data not shown).

Dose response of *lasB* expression. To further investigate the effect of the chosen substances on QS, we employed the *lasB-gfp*(ASV) QS monitor (23) harbored by PAO1. *lasB* is a type IV QS-controlled gene (48), which is maximally induced in the transition between exponential and stationary phases. This induction is reflected in a burst of green fluorescence. The presence of a QSI compound reduces this induction (23). As we only had a crude estimate of the QSI and subgrowth inhibitory concentrations from the selector assays, several different concentrations were tested. To determine the maximal concentration at which growth is not affected but maximum inhibition of the QS-regulated gene fusion is achieved, a 2-fold dilution series of each extract was incubated with a 100-fold dilution of an overnight culture of the QS monitor strain. Growth and fluorescence were monitored over time (Fig. 3A). During growth of this strain, *lasB-gfp* was induced more than 10 fold; however, when garlic extract was added to the growth medium, the onset, synthesis rate, and maximum induction were reduced in a concentration-dependent manner. At a concentration of 2% (vol/vol), the garlic extract significantly reduced the synthesis rate of GFP and lowered induction by about twofold. Usually our garlic extracts did not influence growth when used at concentrations below 1 to 2% (vol/vol). This indicates that garlic extract at this concentration interferes with the QS-controlled induction of *lasB* (Fig. 3A). We noted that differences in age (since picking), origin, and possible subspecies of garlic affect the dose-response relationship. Such batch variations make calibration of each extract obligatory. For 4-NPO, we found 100 μM to be the optimum concentration without affecting growth (Fig. 3B).

Several QSI compounds are present in crude garlic extract. Crude toluene extract of garlic was spotted onto a reversed-

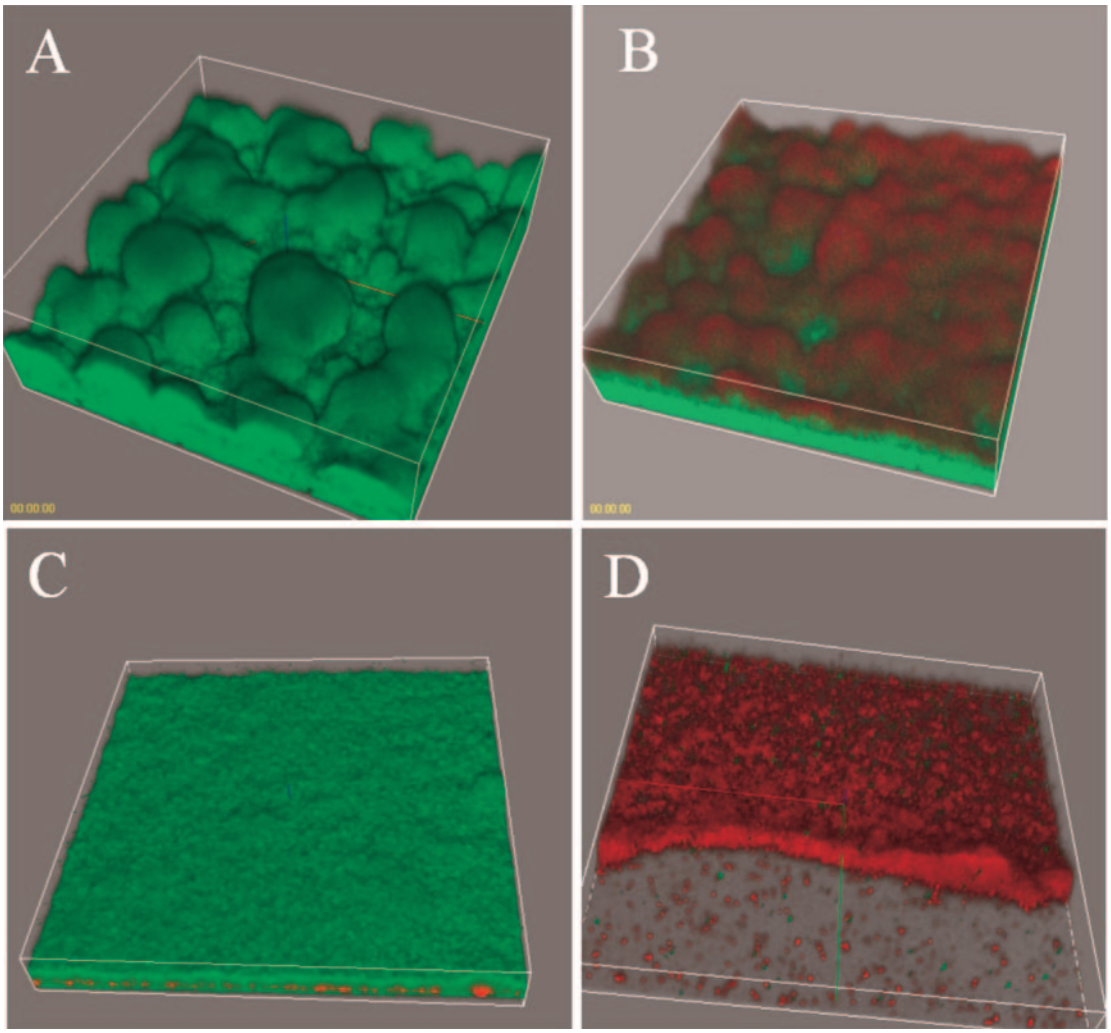

FIG. 5. Three-dimensonal images of 3-day-old PAO1 biofilms grown in the presence (bottom) or absence (top) of garlic extract. The biofilms are either untreated (left) or treated with tobramycin for 24 h (right). Bacterial viability was assessed with the BacLight viability stain: green (live)/red (dead).

phase TLC plate and developed in two dimensions, after which the TLC plates were cast into agar containing QSIS1. After incubation, three spots of QSI activity were observed, indicating that the extract contained more than one biologically active QSI compound (Fig. 4A). This assay was performed with the crude extract retaining the growth inhibitory compound(s). The no-growth area suggested that this QSI activity comigrated with growth inhibitory activity. TLC separation performed on the aqueous phase revealed the presence of only one compound with QSI activity (Fig. 4B). This compound did not block growth in the tested concentration.

DNA microarray analysis. With QSIS and promoter fusions, the QSI compounds were only tested with a few QS-regulated genes. To gain more comprehensive information about the target specificity, the effect of two different garlic extracts (G1 and G6) as well as the pure compound 4-NPO on the transcriptome of *P. aeruginosa* was assessed by Affymetrix DNA arrays. Cultures of PAO1 were brought into exponential growth and at OD_{600} of 0.7, cultures were treated with either 2% (vol/vol) garlic or 100 μM 4-NPO. Although induction and repression of QS-controlled genes formed a continuum throughout the growth cycle (40), a majority of the genes are maximally regulated at the transition to stationary phase (25); hence, growth was followed to OD_{600} of 2.0, at which point samples for array analysis were taken. Absolute expression values from treated cultures were compared to the corresponding values of the untreated control. Expression values from genes estimated not to be present by using the Micro Array suite software, version 5.0, were not included in the analysis. Changes in gene expression were reported as simple fold changes; a change in expression of less than fivefold has proved to be insignificant in previous studies (25, 41). Genes that were significantly (>5-fold) downregulated are listed in Table 2, whereas significantly upregulated genes are listed in Table 3. Performed under these restrictions, the analysis demonstrated that the two garlic extracts affected the expression of 167 (3%) genes in total (Tables 2 and 3). According to our previous mapping (25), 34% of the QS regulon was downregulated by garlic treatment. Affected genes were found among LasR-controlled genes (8% of total number of LasR-controlled genes

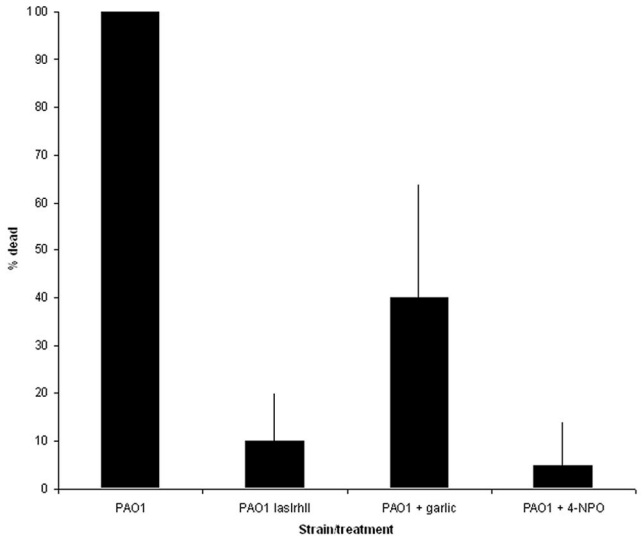

FIG. 6. Mortality of *C. elegans* nematodes living on a lawn of *P. aeruginosa*. Bars show an average of five experiments, and errors bars indicate the standard deviation between experiments.

(25), RhlR-controlled genes (34% of total RhlR-controlled genes) (25), and LasR- plus RhlR-controlled genes (62% of total LasR- and RhlR-controlled genes) (25), indicating that garlic treatment was less efficient against LasR-controlled gene expression. 4-NPO showed a broader spectrum of target genes, as it affected expression of 337 (6%) genes in total; 323 were downregulated and 14 were upregulated (Tables 2 and 3). A total of 37% of the QS regulon was found to be downregulated by this treatment. Affected genes were found among LasR (13%), RhlR (42%), and LasR plus RhlR (67%) genes. This indicates that the garlic compounds and 4-NPO preferentially target the RhlR receptor. When analyzed under similar restrictions, the well-established QSI, furanone compound 30, was found to inhibit 46% of the QS-regulated genes in *P. aeruginosa* PAO1. Affected genes were found among LasR (23%), RhlR (84%) and LasR plus RhlR (39%) genes. Of the genes regulated by garlic extract and 4-NPO, 53 and 49%, respectively, are of unknown function. This seems reasonable, as about 44% of the genes in the PAO1 genome have unknown functions (25).

The two garlic extracts and 4-NPO have 111 target genes in common. Genes such as *lasA* and *lasB* (encoding elastase protease), *rhlAB* (encoding rhamolipids), and *chiC* (encoding chitinase), as well as *aprA*, *phzA1B1*, *phzS*, *phzC2D2E2F2G2*, and *PA1L*, were downregulated by the garlic extracts and 4-NPO. All these are involved in virulence and pathogenesis of *P. aeruginosa*.

The genes of *P. aeruginosa* have been divided into several functional classes (by PseudoCAP) (25). Garlic extract has a preference for genes belonging to the secreted factors (toxins, enzymes, and alginate) group, targeting 11 genes (22%) of that group. Similar results are seen with 4-NPO, as it targets 13 genes from that group. This correlates with the fraction of secreted factors (toxins, enzymes, alginate) genes regulated by QS. QS regulates 11 genes (22%) from this group (25), 10 of which match the genes targeted by garlic and 4-NPO. As rhamnolipid, phenazine, and other virulence factors are members of this group, it suggests that the garlic extracts can downregulate virulence of PAO1.

The garlic extract used in this transcriptome analysis is still a relatively crude extract containing a mixture of compounds. This may account for the effects on non-QS-regulated genes. However, it is fair to assume that the pure compound in the optimum concentration will exert a much more pronounced effect and have a higher specificity on the QS regulon. 4-NPO (which is a pure compound) downregulated a significant portion of the QS-regulated genes. However, it also affected expression of a number of non-QS-regulated genes. Together with the fact that 4-NPO is required at a 10-fold-higher concentration than furanone compound 30 (25), it indicates that 4-NPO is less efficient in affecting the QS regulon than the furanone compound.

Effect on biofilm tolerance to tobramycin. It has previously been shown that *P. aeruginosa* biofilm cells are highly tolerant to antibiotic treatment (3). Davies et al. (9) demonstrated that a QS mutant of PAO1 is more susceptible to sodium dodecyl sulfate (SDS) treatment than the wild-type counterpart. Further, Hentzer et al. (25) showed that biofilms treated with a QSI became susceptible to both SDS and tobramycin. We tested if garlic extract exhibited a similar effect. Two sets of PAO1 biofilms were allowed to form for 3 days in flow chambers in which the medium contained either no garlic or 1% garlic. Following this, each biofilm was challenged with 340 μg of tobramycin/ml for 24 h. The effect of the antibiotic treatment was assessed by live/dead staining. In Fig. 5D, it can be seen that most of the cells in the biofilm treated with both garlic and tobramycin died (Fig. 5D). In striking contrast, only the cells in the top layer of the biofilm treated with tobramycin alone were killed (Fig. 5B). Further, Fig. 5C shows that treatment with garlic extract alone had no effect on biofilm viability. Comparing the untreated biofilm (Fig. 5A) with a garlic-treated one (Fig. 5C), a difference in architecture is observed. The untreated biofilm exhibited the classical mushrooms of a *P. aeruginosa* biofilm grown in glucose-containing medium. A flatter, undifferentiated biofilm was formed when the medium contained 1% garlic extract. This effect would be expected by QSI treatment, since a QS-defective *lasI* mutant of PAO1 has been found to form flat, undifferentiated biofilms (9).

***C. elegans* nematode model.** Previous work has shown that *P. aeruginosa* PAO1 kills *C. elegans* when the strain is grown on brain heart infusion (BHI) agar (17). However, a *lasR* mutant is strongly attenuated in this virulence model (17). Thus, the presence of a QS blocker in the medium is expected to abolish the ability of *P. aeruginosa* PAO1 to kill *C. elegans*. In agreement with a previous study (17), we observed that when *C. elegans* was placed on a lawn of *P. aeruginosa* PAO1 grown on BHI plates, the nematodes were killed within a few hours (Fig. 6). However, when the medium was supplemented with either 2% garlic extract or 100 μM 4-NPO, only 40 or 5% of the worms were killed within 5 h, respectively. As a comparison, a QS-deficient *lasI rhlI* double mutant killed 10% of the nematodes. These results provide strong evidence that garlic extract and 4-NPO reduce expression of pathogenic traits in PAO1.

In conclusion, we have developed recombinant bacteria which can be employed as rapid QSI screens. These QSIS systems have been used to screen a library of pure compounds

and a selection of extracts from food sources and herbal medicine. We have identified a number of active QSI (Table 1) and further investigated the effects of the two most active: garlic extracts, which contain at least three different QSIs, and 4-NPO. Both were able to inhibit QS in a concentration-dependent manner. GeneChip analysis revealed that garlic extract and 4-NPO had a profound effect on genes, especially virulence genes, regulated by QS. Both garlic extract and 4-NPO significantly lowered the pathogenicity of *P. aeruginosa* PAO1 in a *C. elegans* nematode model. In a clinical context, drugs based on QSI compounds might become interesting, due both to the ability to downregulate expression of virulence factors and to the ability to make biofilms much more susceptible to conventional antibiotic treatment.

Work is currently in progress to identify the active QSI component in garlic extract, as well as to investigate the effect of garlic extract in a pulmonary mouse model.

ACKNOWLEDGMENTS

Our work has received financial support from the Danish Technical Research Council, the Villum Kann-Rasmussen Foundation, the Cystic Fibrosis Foundation Therapeutics, Inc., and the Mukoviszidose e.V.

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